Saskatchewan Agriculture, Food and Rural Revitalization

# ADF

## AGRICULTURE DEVELOPMENT FUND

## *FINAL REPORT*

**19990362**

**MODIFICATION OF CANOLA OIL CONTENT BY ALTERING EXPRESSION OF AN OIL ASSEMBLY ENZYME**

**Funded by: The Agriculture Development Fund**

**May, 2003**
**Prepared by: Plant Biotechnology Institute**

105-00328/

# Final Report

## Project # 19990362

**"Modification of Canola Oil Content by Altering Expression of an Oil Assembly Enzyme"**

**Project SADF (Canola Oil Content) 19990362**
**Res. 55BX**

Prepared for the Saskatchewan Department of Agriculture and Food

**Executive Summary:**

We have been successful in completing our assessment of over-expressing DGAT in the model Crucifer *Arabidopsis*, to yield transgenic lines with increased oil/ seed and increased average seed weight. The thrust of the current project has been a parallel assessment of similar transgenics, produced in the canola cultivar Quantum. We are pleased to report that, to date, we have 30 independent single and multiple insert DGAT over-expression lines and these have shown promising results at the $T_2$ level. We have multiplied these selected lines to give $T_3$ seed for field-testing in the summer of 2003 by Dow AgroSciences.

**Background**:

The objective of this project was to develop canola germplasm with elevated oil content by over- expression of the DGAT gene(s). We constructed plant transformation vectors with seed-specific promoters to drive the expression of the *Arabidopsis* or other DGAT genes for genetic transformation of *Brassica napus* canola cv. Quantum.

## Detailed Technical Report

- **OBJECTIVE 1: Analyses of $T_3$ *A. thaliana* DGAT transgenics as a *Brassica* model ACHIEVED**

We have solidly demonstrated that the over-expression of the *TAG1* DGAT (Diacylglycerol acyltransferase) gene in wild-type *Arabidopsis thaliana* has resulted in dramatic increases in average oil content (**Figs. 1 & 2**) and seed weight (**Fig. 3**). These increases were associated with the level of DGAT transcript over-expression (**Fig. 4**).

A manuscript of this important test model data (and a follow-up to our original cloning paper), a world first, was published in *Plant Physiology* during the course of the current project. A copy of this paper is attached in ***APPENDIX II***.

***Arabidopsis Data:***

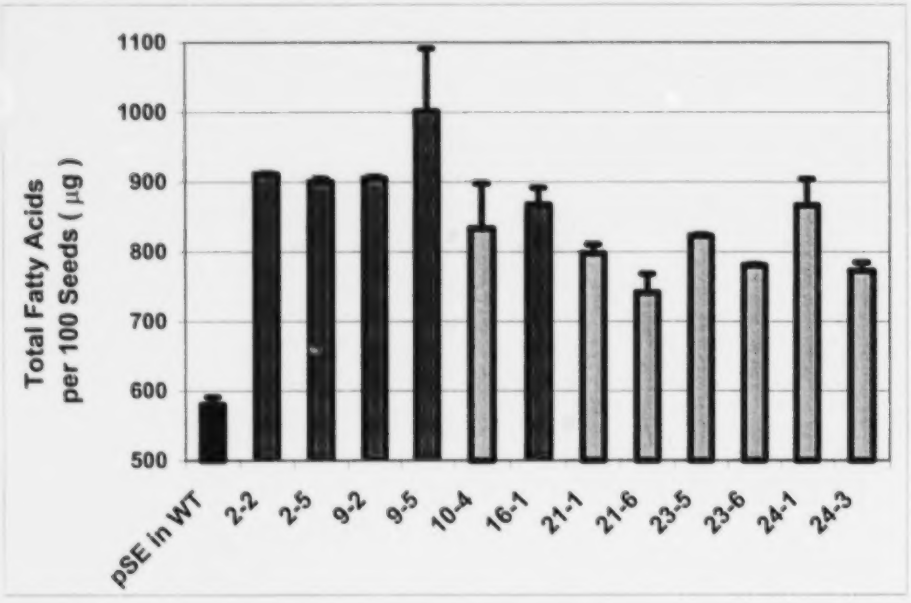



**Fig. 1:** Oil Content (Total µg FAs/ 100 seeds) in Arabidopsis thaliana $T_3$ seed lines. Shown are data for plasmid-only WT, multiple insert (hatched bar) and single insert (grey bar) homozygous transgenic lines.

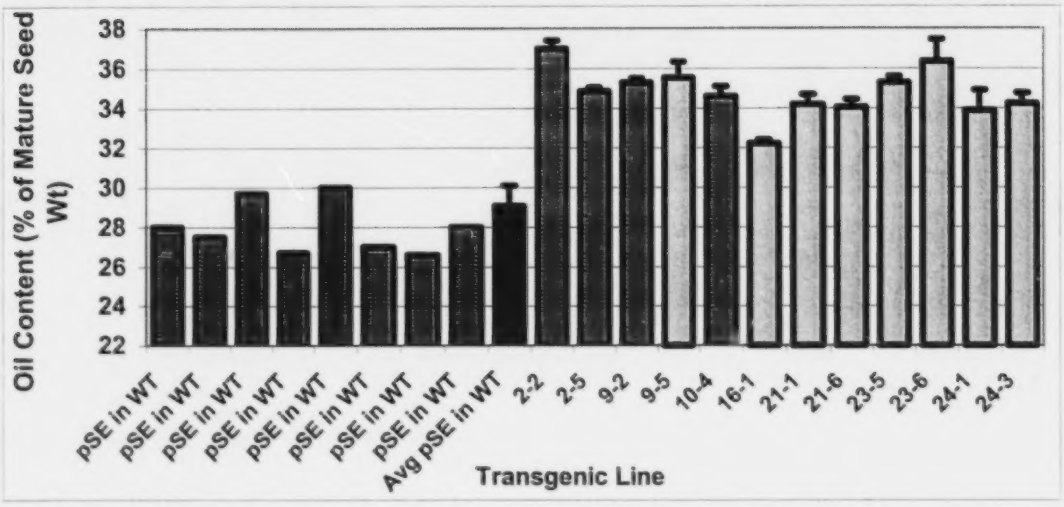



**Fig. 2.** Transformation of wild type (WT) *Arabidopsis thaliana* with the DGAT cDNA under the control of a napin promoter, leads to a higher seed oil content. Homozygous $T_3$ napin:DGAT lines were sampled in triplicate, each sample consisting of 100-200 seeds/ sample, accurately counted and weighed. For the plasmid only pSE in WT transgenic controls and nt-WT and nt-AS11controls, 10 individual transgenic plants were sampled and individual control seed lots similarly analyzed. Each error bar indicates standard error of the means. Seed oil content (oil as a % of mature seed weight) is shown for *A. thaliana* $T_3$ seeds of pSE (empty plasmid) control WT transgenics (stippled bars show representative oil content ranges; solid black bar is the average of oil contents in 10 independent pSE in WT plasmid only controls), nt-WT controls (white bar), nt-AS11 controls (checkered bar), and homozygous napin:DGAT transgenic lines with multiple inserts 2-2, 2-5, 9-2, 9-5 and 16-1(hatched bars) and lines with single inserts 10-4, 21-1, 21-6, 23-5, 23-6, 24-1 and 24-3 (grey bars).

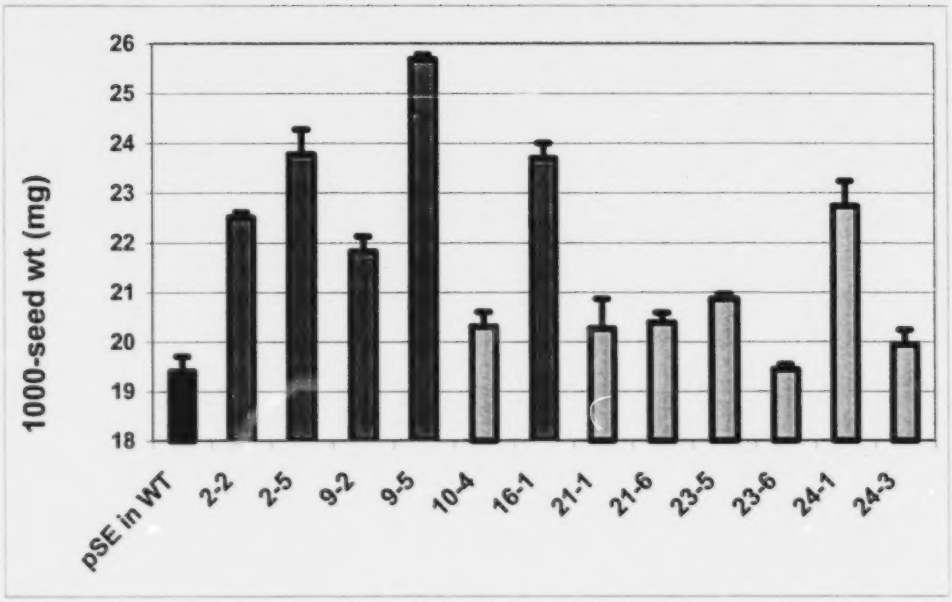



**Fig. 3:** Introduction of the napin:DGAT cDNA results in increases in the average 1000-seed weight (expressed as mg weight / 1000 seeds) of *A. thaliana* $T_3$ seeds. Homozygous $T_3$ napin:DGAT lines were sampled in triplicate, each sample consisting of 100-200 seeds/ sample, accurately counted and weighed. For the plasmid only pSE controls, 10 individual transgenic plants were sampled and individual control seed lots similarly analyzed. Each error bar indicates standard error of the means. Data is presented from pSE in WT (empty plasmid) control transgenics (solid black bar), nt-WT control lines (white bar), nt-AS11 control lines (checkered bar), homozygous napin:DGAT transgenic lines with multiple inserts 2-2, 2-5, 9-2, 9-5, 16-1(hatched bars) and single insert lines 10-4, 21-1, 21-6, 23-5, 23-6, 24-1 and 24-3 (grey bars).

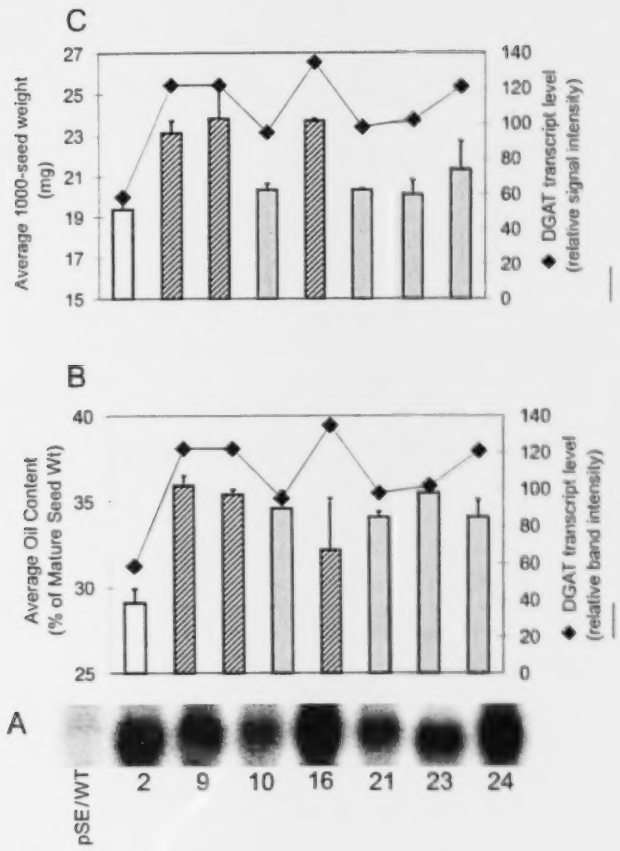



**Fig. 4: A.** Northern analysis of *TAG1* gene expression in pooled developing $T_3$ seed samples from *Arabidopsis thaliana* pSE (empty plasmid) WT control transformants, as well as developing progeny from parent transgenic lines 2, 9, 10, 16, 21, 23 and 24, transformed with the napin:DGAT construct. Total RNA was extracted from siliques containing mid-green developing seeds. The *TAG1* DNA probe was $^{32}P$ labeled by random priming. **B.** Correlation of relative DGAT transcript level (♦) with oil content (% of mature seed weight); pSE control (black bar) or napin:DGAT multiple insert (hatched bars) and single insert (grey bars) transgenics). Values for transgenics represent the average from the corresponding homozygous $T_3$ progeny as reported in Fig. 2. **C.** Correlation of relative DGAT transcript level (♦) with average seed weight (expressed as mg/1000 seeds); pSE control (black bar) or napin:DGAT multiple insert (hatched bars) and single insert (grey bars) transgenics). Values for transgenics represent the average from the corresponding homozygous $T_3$ progeny as reported in Figure 3.

- **OBJECTIVE 2: Transformation and $T_2$ analyses of *B. napus* cv. Quantum *A. thaliana* DGAT transgenics ACHIEVED**

*Transformation:*

Electrocompetent *Agrobacterium* cells, GV3101 (pMP90) strain, were prepared as follows: An *Agrobacterium* culture was grown 24 to 48 hrs in 2YT, and when the absorbence at 600 nm reached 0.5 to 0.7, the cells were chilled on ice and pelleted by centrifugation (5,000 *x g*, 10 min in a GSA rotor at 4°C). The pellet was washed in 1, 0.5 and 0.02 volumes of cold 10% sterile glycerol and resuspended in 0.01 volume of cold 10% glycerol. The electrocompetent cells were then frozen in liquid $N_2$ and stored at -70°C. The *Agrobacterium* cells were transformed by electroporation with 20-50 ng of transforming DNA (napin:DGAT) according to the manufacturer's instructions, plated on a selective medium (LB with 50 μg/ml kanamycin) and incubated overnight at 28°C. Single transformed cells were grown overnight (28°C, 225 r.p.m.) in 5 ml LB with 50 μg/ml kanamycin and 25 μg/ml gentamycin. DNA extraction and purification were performed with a Qiaprep Spin Miniprep kit (Qiagen). The fidelity of the construct was re-checked by DNA sequencing before plant transformation.

Transformation experiments have been undertaken to transfer the napin:DGAT construct into *B. napus* cv. Quantum *via Agrobacterium*-mediated transformation protocols (Moloney et al., 1989 with modifications introduced at PBI/NRC). Seeds of cv. Quantum were surface-sterilized by submerging them in a solution containing 70% bleach and 0.01% Triton X-100 for 15 min with occasional agitation, followed by four rinses with sterile water. Sterilized seeds were then plated on ½ strength hormone-free MS with 1% sucrose. Plates were then kept at 22°C under fluorescent illumination (120 $\mu E \cdot m^{-2} \cdot s^{-1}$) under a 16 h light/8 hour dark regime. A 5 ml *Agrobacterium* suspension in LB medium containing 50 μg/ml kanamycin and 25 μg/ml gentamycin was cultured overnight at

22°C. Cotyledons from four-day-old seedlings were cut and only the petioles dipped in *Agrobacterium* solution. The inoculated explants were plated in medium containing Murashige minimal organics with 3% sucrose, 4.5 mg/L benzyl adenine, pH 5.8, solidified with 0.7% phytagar. Sealed plates were kept at 22°C under fluorescent illumination (120 $\mu E \cdot m^{-2} \cdot s^{-1}$) under a 16 h light/8 hour dark regime at the shelf. After 3 days of cocultivation, explants were transferred to a medium containing Murashige minimal organics with 3% sucrose, 4.5 mg/L benzyl adenine (BA), pH 5.8, 0.7% phytagar and 300 mg/L timentin (SmithKline Beecham). After a further 7 days, explants were transferred to plates of medium containing Murashige minimal organics with 3% sucrose, 4.5 mg/L BA, pH 5.8, 0.7% phytagar with 20 mg/L kanamycin and 300 mg/L timentin in deep (25 X 100 mm) petri dishes. Plates were sealed and incubated as before. Promising explants (those showing initiation of formation of kanamycin-resistant green shoot/callus) were transferred to fresh plates of same medium every 14-21 days. In 2-3 weeks on the above medium, shoots began to develop. These regenerants were then transferred to shoot-development medium (Murashige minimal organics with 3% sucrose, pH 5.8, 0.7% phytagar with 20 mg/L kanamycin and 300 mg/L timentin) to grow plantlets with normal shoot morphology. This often took place with a single or multiple transfer to fresh plates of the same medium to obtain a normal looking shoot. When good shoots were generated any unwanted callus growth around the base was cut off and the shoots were transferred to rooting medium (Murashige and Skoog with 3% sucrose, 0.2 mg/L indole butyric acid, pH 5.8, 0.7% phytagar with 20 mg/L kanamycin and 300 mg/L timentin) in tall jars (e.g. 4 oz baby food jars) for rooting. Once a good root system was formed, these putative transformants were removed from jars and transferred to moist potting soil. Plants were kept covered to maintain high humidity which helped them to harden.

As per the scheme below, to date we have produced **30** confirmed independent *B. napus* cv. Quantum-based DGAT transgenic plants (events). Leaf samples were taken to extract DNA and were used for PCR work for the confirmation of the transgenic genotype of these regenerants. At present,

we are in the process of identifying and multiplying homologous seed lots for all 30 DGAT transformants. By late summer we should be assessing the homologous seed lots for oil quantity and seed weight. **We believe that out of these 30 transformants, we should be able to identify 5 – 10 independent transgenics where plant performance is not negatively affected because of the positional effects of the insertion of the DGAT gene(s) in the *B. napus* genome.**

**A SCHEMATIC DIAGRAM FOR THE DEVELOMENT OF DGAT TRANSGENIC LINES IS AS FOLLOWS:**

Total number of DGAT transformants ($T_0$) identified through PCR to date- **30**

**Primary B. napus transgenic plant** $T_0$ May, 2001

R_ (Hemizygous genotype)

Selfing

**(RR) (R_) (_ _)** $T_1$ Sept., 2001

Res. Res. Sus.

*Segregation analysis* on kanamycin plates* $T_1$ Oct., 2001

10 plants from each line transplanted to soil $T_1$ Dec., 2001

These plants were self-pollinated and harvested individually $T_2$ May, 2002

*Segregation analysis on kanamycin plates* $T_2$

Jun-Jul, 2002

No segregation will indicate that the parent plant has **RR (homozygous)** genotype while **R_ (heterozygote)** type will show segregation

**DGAT assay to be done on single & multiple insert lines and best lines identified. Antibody analysis (Western blots) complemented this**

Evaluation of oil quantity and seed weight on the homozygous genotype $T_2$
Sep, 2002-Mar, 2003

↓

Seed multiplication for Dow Field Trials $T_3$
Jan- Mar, 2003

*Screening Transformed ($T_1$) Brassica napus Seeds (Segregation Analysis)*

*Seed Sterilization:*

1) Transfer ~ 100 seeds in a 2ml tube (fill it with seeds upto 750ul mark).

2) Add 1ml of 95% Ethanol, shake for 15sec and carefully pour off liquid.

3) Add 1ml of sterilization solution (70% Javex + a drop of Tween 20), put on shaker for 20 min and carefully pour off liquid.

4) Rinse seeds several times with sterile dd $H_2O$. Dry in petri dish in the flow hood.

*Seed Germination:*

5) Grow out ~ 100 seeds in Majenta jars. To carry out this just plate seeds in 60 x 15mm petri dishes containing ½ strength hormone-free MS with 1% sucrose and place the dish in the pre-sterile jars. Incubate jars at $25^0$C in 16hr photoperiod for 4-5 days.

6) After 4-5 days of growth, cut-off the root part and stick the apical meristem part in the in 60 x 20mm petri dishes containing rooting media[a] (M5) + 20 mg/L kanamycin[b] selection and place the dish in the pre-sterile jars. Incubate jars at $25^0$C in 16 hr photoperiod.

7) In ~ 2-3 weeks time, kanamycin-resistant seedlings will have root system developed. Record the number of plants having roots (+) *versus* those which are dead or without any root development (-) to yield a segregation ratio analysis.

8) Wash the media off of ~ 10 well rooted/vigorous plants and transplant them in pots filled with Redi-earth; cover them with plastic jars for a week or so for hardening to take place.

9) Grow out each plant to maturity and collect the self-pollinated $T_2$ seeds individually for further analysis.

[a]**Rooting media**

| | ½ L | 1L | 2L | 3L |
|---|---|---|---|---|
| MS/B5 | 2.29g | 4.44 | 8.88g | 13.32 |
| Sucrose 3% | 15g | 30g | 60g | 90g |
| NAA (0.1mg/L) | 100ul | 200ul | 400ul | 600ul |
| pH 5.8 | | | | |
| Agar 0.7% | 3.5g | 7g | 14g | 21g |

**Autoclave and add kanamycin** (20mg/l)

[b]For 500ml of media add **200ul** of 50mg/ml kanamycin.

*Results of $T_2$ Seeds Analyses:*

$T_2$ seeds from all 30 transgenics were screened for fatty acid profile and oil content using the FAME (fatty acid methyl ester) analysis routinely performed in our lab. The highlights of this study (selected DGAT-over-expression lines) are summarized in **Tables I & II** and **Figs. 5-7**.

***Table I: Seed Weights and % Seed Oil in Selected $T_2$ DGAT Over-expression Lines***

| Line # | A: Wt/ 100 seeds (mg) | B: Seed Oil Content (% of Dry Wt) |
|---|---|---|
| Quantum | 306.2 | 37.3 |
| 7a-2 | 257.9 | 40.0 |
| 11a-9 | 318.5 | 35.0 |
| 43-1* | 318.5 | 38.4 |
| 49a-1 | 280.3 | 38.0 |
| 5a-2* | 389.5 | 36.8 |
| 12a-3 | 469.4 | 31.0 |
| 17a-3 | 472.1 | 31.9 |
| 23-1 | 472.2 | 30.1 |

*2 inserts

*Table II: Oil Content (mg oil/ 100 seeds) in Selected $T_2$ DGAT Over-expression Lines*

| Line # | mg oil/ 100 seeds<br>= [A x B] / 100 | % increase |
|---|---|---|
| Quantum | 114.2 | |
| 7a-2 | 103.2 | |
| 11a-9 | 111.5 | |
| 43-1* | 122.3 | 11.0 |
| 49a-1 | 106.5 | |
| 5a-2* | 143.3 | 25.5 |
| 12a-3 | 145.5 | 27.4 |
| 17a-3 | 150.6 | 31.9 |
| 23-1 | 142.1 | 24.4 |

*2 inserts

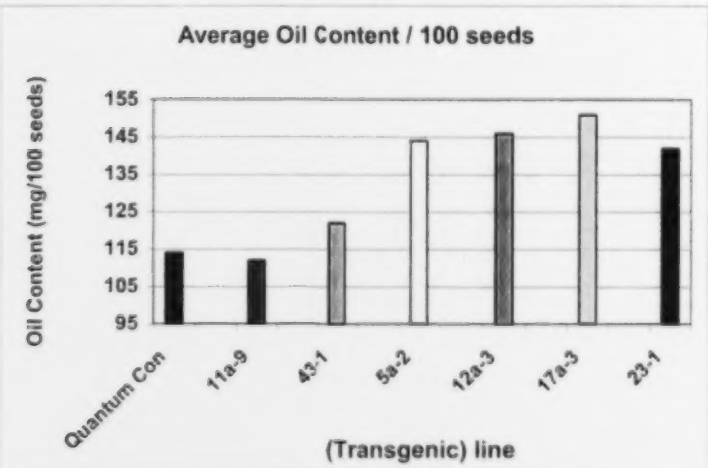



**Fig. 5**

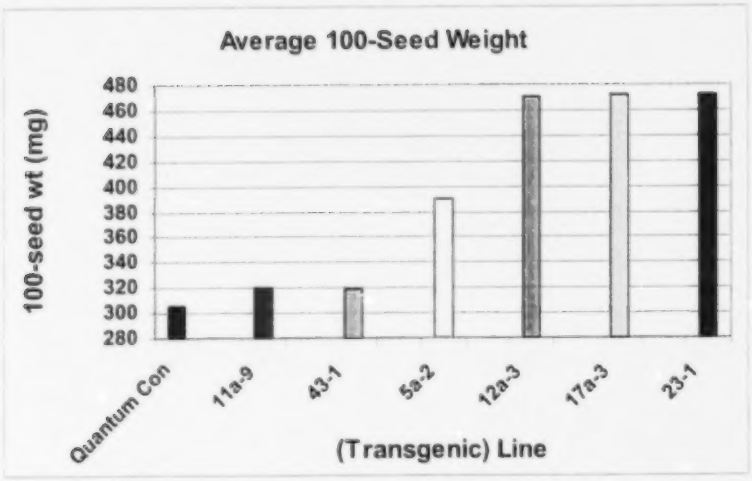

Average 100-Seed Weight
100-seed wt (mg)
480
460
440
420
400
380
360
340
320
300
280
Quantum Con
11a-9
43-1
5a-2
12a-3
17a-3
23-1
(Transgenic) Line

Fig. 6

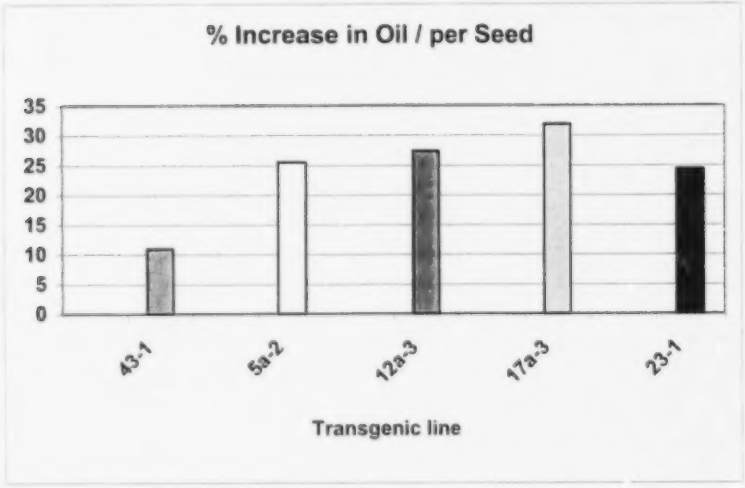

% Increase in Oil / per Seed
35
30
25
20
15
10
5
0
43-1
5a-2
12a-3
17a-3
23-1
Transgenic line

Fig. 7

**Summary of DGAT Over-Expression to Date:**

•Most $T_3$ transgenic lines have an increased seed weight.

•Some transgenics also have increased oil content e.g. Line 43a-1

•The combined increase in seed mass *and* accompanying oil content result in an **overall increase of seed oil (mg) / 100 seeds ranging from 11-31%.**

•The greatest effect of the transgenic expression of DGAT in a seed- specific manner in *B. napus* appears to be an increase in seed weight.

•The relationship between these two seed traits requires further investigation.

**Note: Printouts of the total set of transgenic analyses conducted in *Excel*, are included as hard copies in Appendix III, and as electronic copies on the CD included with this report.**

**Biochemical & Molecular Analyses to be Performed on Field-Trial Material**

**N.B.** The funding for this project ends on March 31 2003, before the field trial results will be assessed (Fall of 2003). We will try to put our in-house technical resources towards this task, but we may also require the assistance of other partners (e.g. Dow AgroSciences, SWP/CanAmera etc.).

*DGAT Assay:* For this assay, protein will be extracted from mid- to late-green seeds of $T_3$ *B. napus* as described in Objective 2 (below). DGAT assays will be conducted at pH 7.4, with shaking at 100 rev/min in a water bath at $30^0$C for 30-60 min. Assay mixtures (0.5 ml final volume) will contain lysate or microsomal protein (250 μg), 90mM HEPES-NaOH, 0.5 mM ATP, 0.5 mM CoASH, 1mM $MgCl_2$, 200 uM sn-1,2 diolein in 0.02% Tween-20, and 18 uM [1-$^{14}$C oleic] (specific activity 2 nCi/nmol) 18:1-CoA as the acyl donor. The $^{14}$C labeled TAGs will be isolated by TLC on silica gel G plates developed in hexane:diethyl ether:acetic acid (70:30:1 v/v/v), the radiolabelled TAG bands to be visualized on a Bioscan AR-2000 radio-TLC scanner using Win-Scan 2D software (Bioscan Inc., Washington DC, USA). The bands will be scraped and quantified as per in-house laboratory procedure.

*Seed Oil Composition and Content Analyses:* The total fatty acid content and acyl composition of seed lipids was determined by FAMEs (fatty acid methyl esters) analysis using a GC with tripentadecanoin (tri-15:0) and 17:0 free fatty acid, as internal and external standard, respectively, as described below:

*Brassica napus) TLE (Total Lipid Extract) preparation:*

Weights and volumes adjusted as per number seeds.

* All glassware is prewashed in Dichloromethane, 2 x, caps in Methanol, 2x

1) Count and weigh 12 seeds/rep and record number and weight. (12 WT Quantum seeds weigh about 30 mg) (Wt. is recorded for calculation of Total oil content (% of DW)).
2) Add 3.0 ml 2: 1 Chloroform: Isopropanol + BHT (Butylated Hydroxytoluene) ( 0.2 % ) containing 500 μg tripentadecanoin (tri-15:0) as an internal standard. Use 16 mm culture tubes, (Pyrex 9826-16) grind with small PRO 200 grinder for 45 seconds.
3) Wash grinder 2 x with 3.0 ml 2: 1 Chloroform: Isopropanol + BHT (0.2%) between samples.
4) Centrifuge at 2,500 rpm for 5 minutes.
5) Optional: Remove supernatant (TLE) with Pasteur pipette and transfer to a clean culture tube. Filter if necessary with a peace of glass fiber filter in a Pasteur pipette.

*Transmethylation:*

6) Add 100 μl of supernatant (TLE) to a clean culture tube.
7) For developing seeds add CBM (1:1:1, Chloroform: Benzene: Methanol) to remove any moisture.
8) Dry under $N_2$ (heating Block at 40 °C ).

9) Add 2 ml of 1% $MeOH/H_2SO_4$ cap tightly and heat at 80 °C for 1 hr in heating block.

10) Stop reaction by cooling tubes in an ice bath to relieve pressure; then add 2 ml of 0.9 % aqueous NaCl, and extract with 1 ml hexane.

11) Mix (vortex) and centrifuge at 2500 rpm for 2 minutes.

12) Remove upper organic layer (hexanes), to a clean culture tube using a Pasteur pipette. Avoid transferring any of the aqueous layer if possible.

13) Extract once more with hexane and combine hexanes extracts.

14) To remove all the water from the extracts, add 1 ml CBM, vortex if cloudy add more CBM, dry under $N_2$ (Heating block at room temp).

15) Redissolve in 100 µl (13.35µg) *External Std* C17:0 Me (Methyl Ester) (stock solution) 0.1335 mg /ml in hexanes. Transfer to a GC vial with insert.

Concentrate (10 µl minimum.) sample under $N_2$ to increase GC sensitivity if required.

*Gas Chromatography*

16) Inject 2 µl on 30 m DB23 column by 0.32 mm i.d. Split injection. Using a temperature gradient of 160 ° C for 1 min, 4 ° C / min to 240 ° C, hold for 10 min. Injection port temp = 200 ° C, Detector = 240 ° C, Helium 24 cm /sec measured at 150 ° C.

17) From peak areas calculate the Wt %, Mol %, mg of oil and % oil using the in-house MS excel spreadsheet (U:\smog\dlb\2003\GC-FAME-New 2003-Temp.xls, and Macro03.xls.).

*Seed Oil and Protein Analyses:*

For the DGAT field trial progeny, a near-infrared reflectance (NIR) method will be used to estimate oil and protein content based on AOCS (American Oilseed Chemist Society) Procedure Am 1-92 using the NIR System 6500 (Foss North America), with soft ware packages NEWISI and WINISI (Infrasoft International LLC). The sample size for NIR scanning will be about 4.5g, enough to fill the ring cup.

The oil and protein contents as determined by NIR, will be calibrated against data obtained from NMR and Leco Protein Analyzer/Kjeldahl analyses (performed with a standard set of Quantum seed samples).

*DNA and RNA isolation from Transgenic DGAT Lines for Southern and Northern Analyses:*
Genomic DNA will be isolated from individual $T_3$ plants following the in-house protocol. A PCR amplification with the primers DGATXbaI (CTAGTCTAGAATGGCGATTTTGGA) and DGATXhoI (GCGCTCGAGTTTCATGACATCGA) will be performed to confirm the presence of the cDNA in the $T_3$ transformants. The PCR profile will be as follows: 94°C 1 min; 30 cycles of 94°C 30 seconds, 55°C 30 seconds, 72°C 1 min; and 72°C 5 min. Southern analyses will be performed to reconfirm and select those transformants containing single or multiple copies of the inserted fragment. For this DNA samples will be digested with restriction enzyme BglII, resolved by electrophoresis on a 1% agarose gel, and Southern blotting performed using a nylon filter (Hybond-N+, Amersham) according to in-house procedure. The DGAT cDNA fragment, labelled with $\alpha$–[$^{32}$P] dCTP (NEN/DuPont) using the Random Primer DNA labelling kit (Gibco BRL), will be used as a probe. Hybridization will be performed at 60°C according to standard protocols. The filter will then to be exposed to Kodak X-OMAT-AR film.

Northern analyses of DGAT transcripts in both control and transgenic lines will be performed as follows: Using the in-house method, total RNA to be extracted from developing $T_3$ seeds at the mid-green stage of development. RNA samples will be denatured with formaldehyde and separated on 1.2 % formaldehyde-agarose gels. 10-40 μg of total RNA to be loaded, and the amount of RNA per lane will be calibrated by the ethidium bromide-staining intensity of the rRNA bands. The DGAT DNA probe will be $^{32}$P labeled by random-priming according to protocols of the manufacturer (BRL) and the blots to be hybridized with $^{32}$P-labeled *Arabidopsis thaliana DGAT* cDNA under stringent conditions

(60°C). Relative intensities of hybridization signals in autoradiograms from northern analyses will be measured with an Ultra Lum Electronic Dual Light™ Transilluminator (Ultra Lum, Inc., 6421 East Alondra Blvd., Paramount, CA 90723-3759) using Scion Image software (Scion Corporation, Frederick, MD, 21703).

- **OBJECTIVE 3: Expression of high antigenic DGAT peptides in rabbit to raise its polyclonal antibody. ACHIEVED**

We have identified five highly antigenic peptide sequences which were injected in rabbits to raise polyclonal antibodies. These antibodies were used in screening developing seed of transgenic lines to assess the level of DGAT protein expression. The following specific peptides (**Fig. 8**) were generated by the Plant Biotechnology Institute's Peptide Synthesis Service. They were combined to form a "mixed cocktail" of antigenic peptides for raising antibodies through the University of Saskatchewan Animal Care Unit.

RRKSRSDSSNGLLLSGSDNNSPSDDVGAPADVRDRIDSVVN AK_Peptite Seq 1

RIDSVVNDDAQGTANLAGDNNGGGDNNGGGRGGGEGRGNA AK_Peptite Seq 2

GGDNNGGGRGGGEGRGNADATFTYRPSVPAHRRARESPLS AK_Peptite Seq 3

PRSACIRKGWVARQFAKLVIFTGFMGFIIEQYINPIVRNSK AK_Peptite Seq 4

FYKDWWNAKSVGDYWRMWNMPVHKWMVRHIYFPCLRSKI AK_Peptite Seq 5

**Fig. 8**. Selected DGAT peptide sequences used in antibody work.

*DGAT Antibody Work:*

Protein from green seeds of non-transformed and transgenic *B. napus* was extracted and run on an SDS-PAGE gel followed by Western blotting as described below.

Rabbit anti-*Arabidopsis* DGAT-Antibody was used for hybridization in western blots. A typical result, as shown in **Fig. 9** below, revealed lines enriched in DGAT expression ( e.g. 12a and 17a) at the protein level. The western trends corresponded to those results obtained by chemical analyses.

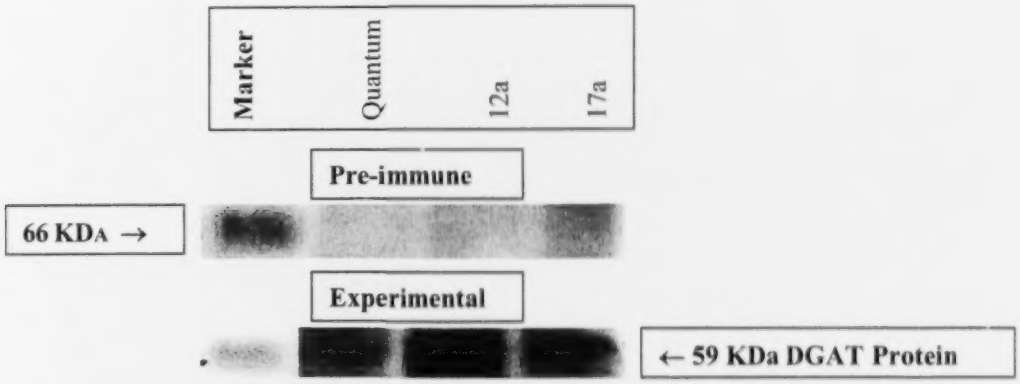



**Fig. 9**

Thus, in the future we have the capability for testing and quantifying the relative DGAT expression in any population. This will allow an early indication of the most promising lines to follow (e.g. in field tested plots when siliques are at mid-development).

*DGAT Protein Extraction, SDS PAGE Gel and Western Blot Analysis:*

*Brassica napus*: Will require protein from non-transformed and transgenic green seeds.

1) Pour 1 mg of green seeds to cold mortar, grind seeds into a fine powder with liquid $N_2$ , transfer to a 2 ml micro centrifuge tube followed by redissolving in 1.5 ml of grinding buffer E (125 mM Tris HCl pH 8.8, 1% (w/v) SDS, 10% (v/v) glycerol, 50mM $Na_2S_2O_5$ . The Plant Journal 1999, 20(2) 251-257). Keep on ice.

2) Once all the extracts are prepared, the tubes are warmed to RT (~20C) to resolubilize the SDS, micro centrifuge at maximum speed (10 min, 13000g) and the supernatant retained. NB: The presence of 1% (w/v) SDS in the sample is compatible with BioRad Dc Protein Assay.

3) Protein Quantification: prepare samples as follows

5 (or 10 this amount can vary) µl protein + 95µl Grinding buffer + 5ml BioRad dye – in triplicate (use 100ul grinding buffer E + 5ml BioRad dye as bank);

mix, wait 15 in, color of the sample protein should be little darker than blank, NOT dark blue (out of range). Determine the $OD_{595}$ 0.007274, and estimate protein concentration using a standard curve for bovine serum albumin.

*SDS Page Gel:*

4) Set up a Bio-Rad Mini-Protean II cell unit for SDS gel electrophoresis. Ready a pre-casted 12% gel by placing a line indicating the bottom of each well on the front glass plate with a marker and removing the comb and cutting/peeling off the tape at the bottom of gel cartridge. Place the gel in the unit and make sure there is no room left between gasket and cartridge to avoid any leaking.

5) Fill the upper and lower buffer chambers with 1X Tris/Glycine/SDS buffer (from Bio-Rad). The upper chamber should be filled to 1 to 2 cm over the notched plate. Rinse the sample wells with electrophoresis buffer.

6) Prepare the protein sample and protein standard mixtures. Bio-Rad mini gel has 10 wells and can take 30µl of sample. Pipette out protein sample of 10ug into a microfuge tube, add 5ul of the loading buffer Z (125mM Tris-HCl pH 6.8, 12% (w/v) SDS, 10% (v/v) glycerol, 22% (v/v) B-

mercaptoethanol, 0.001% (w/v) bromophenol blue- The Plant Journal 1999, 20(2) 251-257) and adjust volume to 20µl with $H_2O$ if required. Similarly for standard – Rainbow colored marker (RPN756 from Amersham Pharmacia biotech), add 4µl of ladder + 6µl loading buffer Z. Add another conventional marker RPN 2107 3ul ladder + 2µl $H_2O$ + 5µl loading buffer Z. Seal the microfuge tube with parafilm.

7) Denature the samples (ladders come ready to load) in boiling $H_2O$ for 3 to 5 minutes. Cool on ice, spin them down.

8) Load the samples using micro pipette/syringe. Insert the pipette tip through the upper buffer and into the well. The mark on the glass plate will act as a guide. Dispense the sample into the well. Electrophorese samples at 50mA until the dye front reaches the bottom of the gel (~ 1 to 1.5hr). Disconnect the power supply.

9) Disassemble the gel – follow the instructions on the back of Bio-Rad gel. Carefully remove the gel from the lower plate. Cut a small triangle off one corner of the gel so the lane orientation is not lost during staining and drying. Proceed with protein detection.

*SDS PAGE Gel Staining (Not needed if doing a Western Blot)*

10) Prewashing – after electrophoresis, transfer gel to a clean 150x15mm petri dish and rinse 3X, 5 min ea, with ~ 100 to 200 ml dd $H_2O$.

11) Staining – add 20 ml GelCode Blue Stain Reagent (from PIERCE) to the petri dish. Gently shake the petri dish, staining intensity reaches a max. within 1 hr. Do 2-3 washes with dd $H_2O$ for 1-2 hr or leave on a shaker o/n for further colour development to reveal even weak protein bands.

12) Take digital picture of the gel.

*Western Blotting (Protocol based on Amersham Pharmacia Biotech Kit)*

13) Run typical SDS-PAGE gel, remove from glass plates and orientate the gel by removing a corner, allow to equilibrate (soak) in the protein transfer buffer[1] for 15 min.

14) Cut a sheet of Hybond-P membrane to size of gel (wear gloves when handling the membrane), pre-wet the membrane in 100% methanol for 10 sec, wash in dd $H_2O$ 1X for 5 min and then equilibrate in the protein transfer buffer for at least 10 min.

15) Assemble the Bio-Rad Mini Trans-Blot cassette – cut 2 pieces of 3mm Whatman filter paper to same size as transfer pads, assemble the transfer sandwich (follow steps described below and as shown in **Fig. 10**) and soak in transfer buffer.

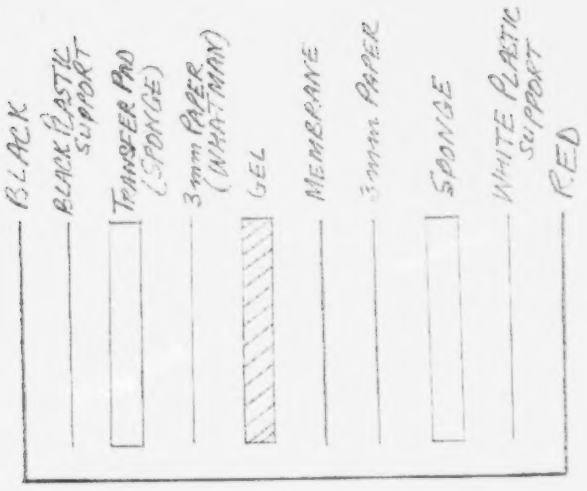



**Fig 10.** Transfer sandwich – assemble in a tray with transfer buffer.

a. Open plastic support and place with black mark (negative side) down.

b. Place a transfer pad and a piece of 3mm paper on top of marked support

c. Place the gel carefully on the top of the filter paper making sure that no air bubbles are trapped between the gel and the paper.

d. Place the membrane on top of the gel making sure that there are no air bubbles. Use transfer buffer, if required, to help remove bubbles.

e. Place the other piece of 3mm paper on top of the membrane and use a test tube like a rolling pin to remove any bubbles which may be trapped.

f. Place second transfer pad on top of 3mm paper and close the sandwich.

g. Place sandwich with hinges up in the trans-blot chamber so that the black marked support is next to the black electrode.

16) Place ~ 1L of transfer buffer and a stir bar in the Bio-Rad Mini Trans-Blot Cell.

17) Transfer for 1.5 hr at 85V with cooling ($4^0$C). This setting is suitable for gels 80 to 320 $cm^2$ in size. It is recommended that cold transfer buffer is used.

18) Following transfer remove the membrane from the blotting cassette, mark the orientation of the gel on the membrane and rinse briefly in TBS[2]. Trim the membrane/blot.

19) Blots may be used immediately or stored. Air dry before storage in between 3mm paper wrapped in Saran Wrap at 2-$8^0$C for up to 3 months. If blots have been allowed to dry out or stored, the wetting procedure (as in step 14) must be repeated.

20) Block non-specific binding sites on the membrane, by immersing the membrane in 5% non-fat dried milk solution (5g milk powder in 100 ml TBS-Tween[3]) for 1hr (blocking can also be done o/n at 2-$8^0$C). Briefly rinse the membrane twice with TBS-T.

21) Incubate with Primary Antibody (DGAT, raised in rabbit) at the optimized dilution in 50 mls TBS-T on a shaker at RT for 1 hr. See attached notes on determination of optimum antibody conc. *Add 16.25 µl of primary AB to 50 mls TBS-T, which is ~3000 fold dilution. Use 5µl/2.5µl of primary AB for10K/20K dilution.*

22) Dump primary AB and briefly wash blot in TBS-T.

23) Incubate with 5µl (or less which equals to 10,000 fold dilution) secondary AB (NA934 – Anti-rabbit Ig, horseradish peroxidase) in 50ml TBS-T for 45 min to 1hr at RT.

24) Dump the secondary AB and briefly rinse in TBS-T with excess wash.

*Detection (ECL Plus System)*

25) Detection reagents (RPN2132 – Amersham Pharmacia Biotech) should be allowed to equilibrate to RT before opening.

26) Mix detection solutions A and B in a ratio of 40:1 (*e.g.* 10 ml solution A + 250 µl solution B). The final volume of detection reagents required is 0.1ml/cm$^2$ of blot.

27) Take solutions, blot, saran wrap, timer, small container, kimwipes and x-ray film/cassette to dark room.

28) Drain buffer from the blot, use kimwipe to remove the last bit and place protein side up on a sheet of saran wrap.

29) Dump the mixed detection reagents directly onto blot and incubate on shaker to assure the solutions are mixed. Cover the whole surface of the blot for 5 min at RT.

30) Drain off excess detection reagent by holding the membrane gently in forceps and touching the edge against a tissue. Place the blot protein side down onto a fresh piece of saran wrap, wrap up the blots and gently smooth away any air bubbles.

31) Place the wrapped blots, protein side up, in a x-ray film cassette. Ensure there is no free detection reagents in the cassette; the film must not get wet.

Switch off lights

32) Place a sheet of autoradiography film on top of the membrane. Close the cassette and expose for 15 seconds.

33) Remove the film and replace with a second sheet of unexposed film. Develop the first piece of film immediately and on the basis of its appearance estimate how long to continue the exposure of the second piece of film. Second exposure can vary from 1 min to 1hr.

- - - - - - -

*Buffers:*

Grinding buffer E (125 mM Tris HCl pH 8.8, 1% (w/v) SDS, 10% (v/v) glycerol, 50mM $Na_2S_2O_5$) For 50ml add: 6.25 ml of 125 mM Tris HCl pH 8.8, 0.5g (w/v) SDS, 5ml (v/v) glycerol, 5ml 50mM $Na_2S_2O_5$ –Sodium metabisulfite.

Loading Buffer Z (125mM Tris-HCl pH 6.8, 12% (w/v) SDS, 10% (v/v) glycerol, 22% (v/v) B-mercaptoethanol, 0.001% (w/v) bromophenol blue. For 20 ml add: 2.5ml of 125mM Tris-HCl pH 6.8, 2.4g SDS, 2ml glycerol, 220 ul B-mercaptoethanol per 1ml of buffer Z at the time of use, 0.02g bromophenol blue. Store in 1ml aliquots up to 6 months at $-70^0$C.

Protein Transfer buffer: For 3 L - 6.639 g CAPS, 300ml Methanol, to pH 11 with NaOH, would probably need 1.5L for small transfer apparatus. Keep at $4^0$C.

Tris Buffered Saline (TBS), pH 7.6 – 2.42 g Tris base (20mM), 8g NaCl, 3.8 ml 1M HCl acid, dilute to 1000 ml with dd $H_2O$, check pH.

10X TBS Tween – 24.2g Tris, 30g NaCl, 10ml Tween 20, adjust pH to 7.6, make to 1L with dd $H_2O$. Store at 2-8$^0$C. Make 1X TBS-T to use in western blotting.

- **OBJECTIVE 4: Isolation and characterization of DGAT homologs from Nasturtium.**

**ACHIEVED**

Subtracted seed cDNA libraries were constructed from target germplasm and then Clontech PCR cDNA subtraction based methods used for library screening/identification and isolation of DGAT homologs. Using a degenerate primer approach a cDNA clone of 1557 bp with 82 % homology to *Brassica napus* DGAT was isolated from nasturtium embryos and submitted to the GenBank (**AY 084052**).

The sequencing data have confirmed the integrity of this DGAT cDNA homolog. We have cloned this into the Baculovirus expression vector (Life Technologies) and the construct sequenced again to check its integrity. Protein from *Arabidopsis* DGAT and Nasturtium DGAT will be harvested from insect cell cultures and will be used in DGAT *in-vitro* assays to compare the activity. *(Status - work to be continued as part of Seed Oil Group research in future)*

We have undertaken a DGAT-related DGAT/ACAT inhibitor work side project which is outlined below:

From a Japanese chemical company, we have obtained samples of the chemical - NTE-122 - an acyl-CoA cholesterol acyltransferase (ACAT) inhibitor, which we hope to use in future studies of DGAT/PDTA.

**PERSONNEL**

Arvind Kumar, B.Sc., M.Sc., a full-time NRC Technician (TO-3) began his tenure on this project on April 1, 2000 with the term ending on March 31st, 2003. Arvind made excellent progress and is a very conscientious and diligent worker who plans his experiments very carefully. The result is that we now have 30 stable transgenic lines which have performed very well in the greenhouse; many lines have elevated oil content/seed and increased seed weight. The true test will be to evaluate these lines in the field.

**INTELLECTUAL PROPERTY:**

**PBI has filed a patent based on our cloning and utility of the DGAT gene to increase oil content and seed weight:**

> **J-T Zou**, **D.C. Taylor**, Yangdou Wei and Colette Jako. "***The Arabidopsis thaliana TAG1 gene Encodes a Diacylglycerol Acyltransferase***". U.S. Provisional Patent Application Ser. No. 60/112,812 (case no. 10974). Priority date: 17/12/98. PCT/CA99/01202 filed 16/12/99. This patent has been filed in the US (09/623,514), Canada (2,355,845), China (99816248.5), Russia (2001117211), Ukraine (2001064118/M), Europe (99960735.1), Australia (17638/00) and India (IN/PCT/2002/0070).

**PROJECT PRESENTATIONS:**

Over the last two years, brief updates on projexct progress have been provided to Dow AgroSciences; Accordingly, they have shown interest in field-testing our best $T_3$ lines in the summer of 2003. Dow is also providing legal advice and assisting NRC-PBI with the costs and in the prosecution of our DGAT patent

**A copy of Dr. Taylor's *PowerPoint presentation* of Dec, 2002, is included on the accompanying CD.**

| | | |
|---|---|---|
| Mr. A. Kumar | Dr. D.C. Taylor | Dr. J-T. Zou |

March 28, 2003

National Research Council Canada
Conseil national de recherches Canada

NRC · CNRC

# Manipulation of DGAT Expression to Improve Oil Quantity and Seed Weight in Canola

*Dr. David C. Taylor*

*Saskatchewan Agriculture and Food*

# DGAT Project - Progress Report

- **Background**
- ***Brassica napus* cv Quantum transformed with DGAT gene (Napin:DGAT:Nos) from *Arabidopsis thaliana* – Seed Wt/Oil data**
- **DGAT Antibody work**
- **Nasturtium DGAT expression examined using recombinant baculovirus system**

# Background

***NOTE:***

- ***This project is currently funded by the SADF.*** We achieved a major goal: the isolation and characterization of the first DGAT gene from plants. Gene identification was facilitated by analysis of gene structure in the AS11 mutant:

- [Katavic et al., 1995; Zou et al, 1999).

- We have a patent position on the DGAT gene and its utility in altering oil content and seed weight (**PCT/CA99/01202; WO 00/36114**).

- **Dow AgroSciences is currently assisting with the patent process**

# Background *contd...*

- Results of DGAT over-expression in the model crucifer *Arabidopsis thaliana*, (a member of the *Brassicaceae* closely related to *B. napus*), indicate that seed oil content can be increased by 3-8% of DW; Fatty acid content increases from the baseline value of 675 μg/100 seeds in empty plasmid controls, to values ranging from 800 to 1000 μg/ 100 seeds in selected $T_3$ transgenic lines. DGAT transgenic $T_3$ seed lines also showed a significant increase in average 1000-seed weight.

- **Jako *et al.*, (2001) Vol. 126, pp. 861-874 *Plant Physiology.*.**

# Background *contd...*

■ **The objective of the project is to build on this proprietary technology to over-express the DGAT gene which encodes an enzyme that may strongly regulate the amount of canola seed oil formed or seed mass.**

■ We utilized the *A. thaliana* DGAT gene directly, and over-expressed in the *B. napus* canola *cv* Quantum, under the control of a seed-specific (napin) promoter.

# Background *contd...*

- We expect that selected transgenic lines will exhibit enhanced oil content and /or improvements in acyl composition (e.g. increased monounsaturated fatty acids, 18:1 c9) or increase in seed weight.

- DGAT genes will also be isolated from species with different fatty acid profiles (e.g. Nasturtium). The structure and the expression profiles of the genes will be compared in relation to oil profile and yield. If a correlation between certain unique gene structure and/or expression specificity can be established, experiments will be actively pursued to develop DGAT as an oil quality *molecular marker*.

# Current Status - *B. napus* Transformation

- Total number of DGAT transformants ($T_0$) identified - 33

**Primary B. napus transgenic plant** $T_0$ May, 2001

R_ (Hemizygous genotype)

⇩ Selfing

**RR** **R_** _ _ $T_1$ Sep., 2001

⇩

*Segregation analysis on kanamycin plates* $T_1$ Oct., 2001

⇩

10 plants from each line transplanted to soil $T_1$ Oct., 2001

⇩

These plants will be self-pollinated and harvested individually $T_2$ Jan., 2002

⇩

*Segregation analysis on kanamycin plates* $T_2$ Mar., 2002 Aug., 2002

⇩

No segregation will indicate that the parent plant has **RR (homozygous)** genotype while **R_ (heterozygote)** type will show segregation

⇩

# Current Status - *B. napus* Transformation contd...

DGAT assay to be done on single insert lines and best lines identified. Antibody analysis (Western blots) will complement this

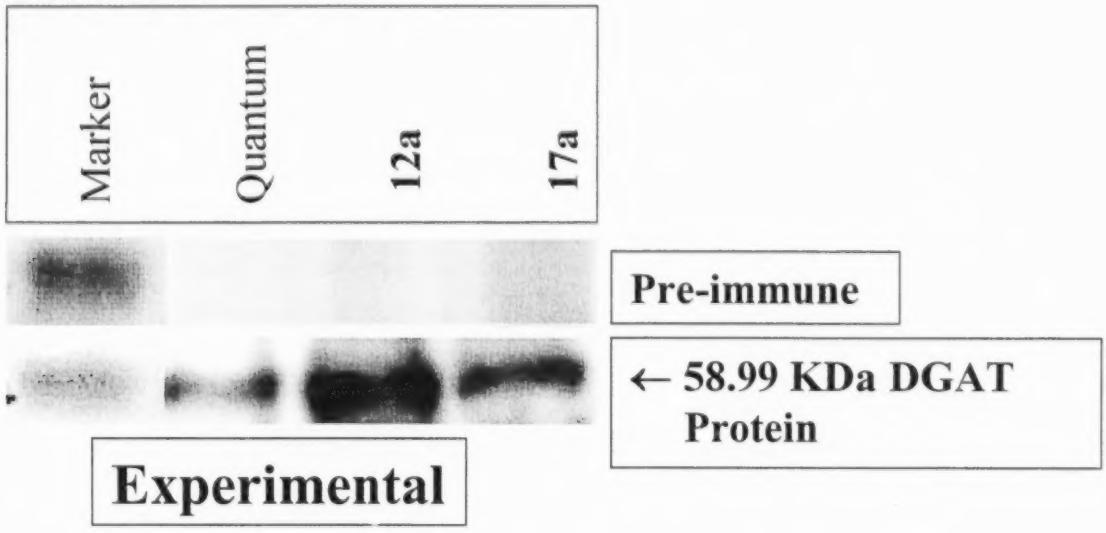
Greenhouse multiplication of the homozygous genotype for

**oil** evaluation $T_3$ Sep.-Dec. 2002

# DGAT Antibody work

- Protein from mid-developing non-transformed seed and from $T_2$ transgenic *B. napus* seed was extracted and used in SDS-PAGE gel followed by Western Blotting.

- DGAT-Antibody raised in rabbit was used for hybridization in Western. Result - successful.




- Now we have capability for testing DGAT in any populations.

# $T_3$ Seed Weight and % Seed Oil

| Line # | Wt/ 100 Seeds mg | Seed Oil % of DW |
|---|---|---|
| **Quantum** | **306.2** | **37.3** |
| 7a-2 | 257.9 | 40.0 |
| 11a-9 | 318.5 | 35.0 |
| **43a-1*** | **318.5** | **38.4** |
| 49a-1 | 280.3 | 38.0 |
| **5a-2*** | **389.5** | **36.8** |
| **12a-3** | **469.4** | **31.0** |
| **17a-3** | **472.1** | **31.9** |
| 23-1 | 472.2 | 30.1 |
| Seed | Seed | Seed |
| *2 inserts | | |

# $T_3$ Seed Weight and % Seed Oil

| Line # | Wt/ 100 Seeds mg | Seed Oil % of DW |
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| **Quantum** | **306.2** | **37.3** |
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| **12a-3** | **469.4** | **31.0** |
| **17a-3** | **472.1** | **31.9** |
| 23-1 | 472.2 | 30.1 |
| Seed | Seed | Seed |
| *2 inserts | | |

# $T_3$ mg oil / 100 seeds

| Line # | mg oil / 100 seeds | % increase |
|---|---|---|
| **Quantum** | **114.2** | |
| 7a-2 | 103.2 | |
| 11a-9 | 111.5 | |
| **43a-1*** | **122.3** | **11.0** |
| 49a-2 | 106.5 | |
| **5a-2*** | **143.3** | **25.5** |
| **12a-3** | **145.5** | **27.4** |
| **17a-3** | **150.6** | **31.9** |
| **23a-1** | **142.1** | **24.4** |
| | | |
| *2 inserts | | |

# $T_3$ mg oil / 100 seeds

| Line # | mg oil / 100 seeds | % increase |
|---|---|---|
| Quantum | 114.2 | |
| 7a-2 | 103.2 | |
| 11a-9 | 111.5 | |
| 43a-1* | 122.3 | 11.0 |
| 49a-2 | 106.5 | |
| 5a-2* | 143.3 | 25.5 |
| 12a-3 | 145.5 | 27.4 |
| 17a-3 | 150.6 | 31.9 |
| 23a-1 | 142.1 | 24.4 |
| | | |
| *2 inserts | | |

# Summary to DGAT Over-Expression to Date

- Most $T_3$ Transgenic lines have an increased seed weight
- Some transgenics also have increased oil content e.g. 43a-1
- The combined increase in seed mass AND accompanying oil content result in an overall increase of Seed oil (mg) / 100 seeds ranging from 11-31%
- The greatest effect of the transgenic expression of DGAT in a seed-specific manner in *B. napus* appears to be an increase in seed weight
- The relationship between these two seed traits requires further investigation

AAFC CNRC

# Overview of scheme to generate Baculoviruses expressing recombinant DGAT

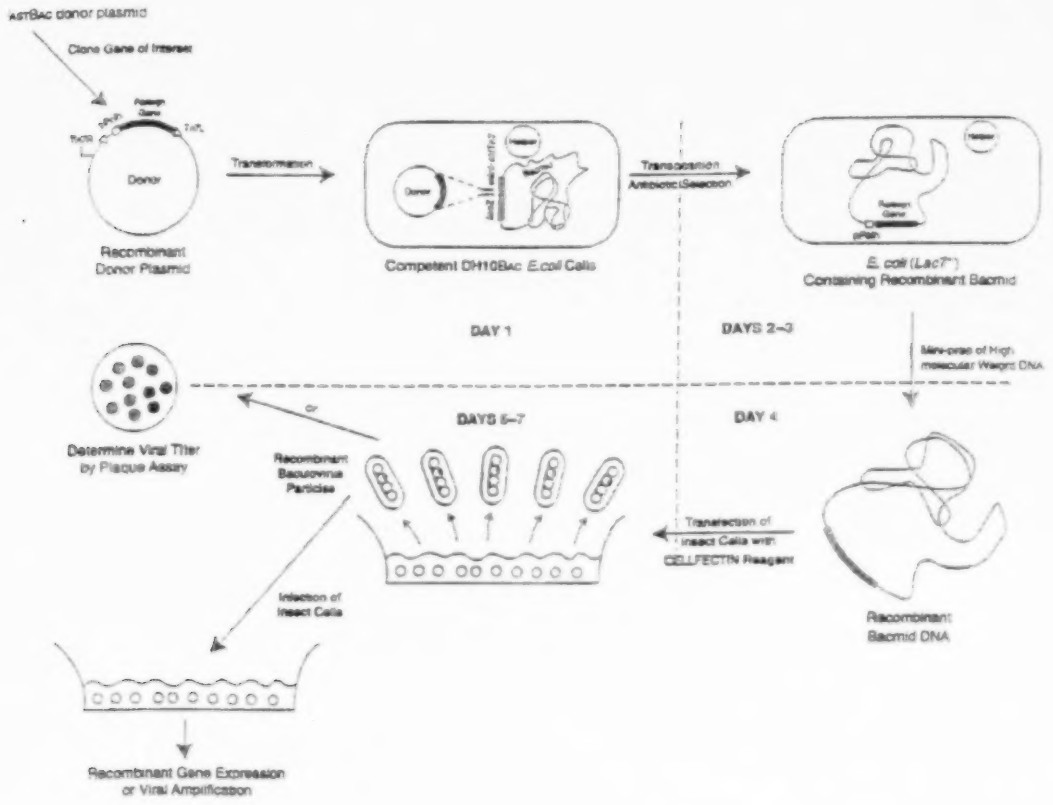


**Figure 1. Generation of recombinant baculoviruses and gene expression with the Bac-to-Bac Expression System.** The gene of interest is cloned into a pFastBac donor plasmid, and the recombinant plasmid is transformed into DH10Bac competent cells which contain the bacmid with a mini-*att*Tn7 target site and the helper plasmid. The mini-Tn7 element on the pFastBac donor plasmid can transpose to the mini-*att*Tn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids are identified by disruption of the *lacZα* gene. High molecular weight mini-prep DNA is prepared from selected *E. coli* clones containing the recombinant bacmid, and this DNA is then used to transfect insect cells.

## This work is in progress

| Run # | Row #, Seed | 17:0 | % Oil | 1000 seed Wt, mg | mg FA's /100 seeds | % 16:0 | % 18:0 | % 18:1 | % 18:2 | % 18:3 | % 20:0 | % 20:1 | % 20:2 | % 22:0 | % 22:1 | % Sats | % Mono | % Poly | Wt % LCFA | Wt % VLCFA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Quantum Control A | kumar100.d | 1.67 | 38.6 | 321.5 | 119.18 | 3.51 | 26.05 | 1.81 | 0.00 | 0.75 | 10.78 | 0.76 | 0.53 | 0.46 | 25.72 | 41.70 | 45.82 | 2.85 | 60.37 | 39.63 |
| kumar101.d | Quantum control b | 1.47 | 39.0 | 291.1 | 108.91 | 3.33 | 1.08 | 22.24 | 15.21 | 9.95 | 0.74 | 11.82 | 0.00 | 0.00 | 0.44 | 5.39 | 68.54 | 26.07 | 53.48 | 46.52 |
| kumar102.d | 3-2a | 1.45 | 28.1 | 342.8 | 92.04 | 4.80 | 1.82 | 57.49 | 21.34 | 8.51 | 0.80 | 1.31 | 0.00 | 0.00 | 0.53 | 7.76 | 62.18 | 30.05 | 96.73 | 3.27 |
| kumar103.d | 3-2b | 1.23 | 27.5 | 305.8 | 80.43 | 4.93 | 1.97 | 58.28 | 20.57 | 7.98 | 0.85 | 1.27 | 0.00 | 0.00 | 0.57 | 8.15 | 63.13 | 28.72 | 96.60 | 3.40 |
| kumar104.d | 4-2a | 1.82 | 30.4 | 370.0 | 107.60 | 4.70 | 2.18 | 62.21 | 18.46 | 6.43 | 0.93 | 1.39 | 0.00 | 0.00 | 0.61 | 8.22 | 66.89 | 24.89 | 96.36 | 3.64 |
| kumar105.d | 4-2b | 1.39 | 31.8 | 382.0 | 116.12 | 4.60 | 2.03 | 62.48 | 18.28 | 6.45 | 0.87 | 1.38 | 0.00 | 0.00 | 0.54 | 7.91 | 66.92 | 25.17 | 96.05 | 3.95 |
| kumar106.d | 5-2a | 1.57 | 31.3 | 275.8 | 82.49 | 4.70 | 1.87 | 60.69 | 18.71 | 7.51 | 0.87 | 1.26 | 0.00 | 0.00 | 0.62 | 7.87 | 65.90 | 26.23 | 96.50 | 3.50 |
| kumar106.d | 5-2a | 1.57 | 31.3 | 275.8 | 82.49 | 4.70 | 1.87 | 60.69 | 18.71 | 7.51 | 0.87 | 1.26 | 0.00 | 0.00 | 0.62 | 7.87 | 65.90 | 26.23 | 96.50 | 3.50 |
| kumar107.d | 5-2b | 1.72 | 32.2 | 262.3 | 80.78 | 4.72 | 1.87 | 60.47 | 18.80 | 7.43 | 0.97 | 1.50 | 0.00 | 0.00 | 0.55 | 7.92 | 65.86 | 26.22 | 96.33 | 3.67 |
| kumar108.d | 6-5a | 1.59 | 30.4 | 296.1 | 86.23 | 4.87 | 1.55 | 57.88 | 21.47 | 7.92 | 0.74 | 1.31 | 0.00 | 0.00 | 0.46 | 7.54 | 62.84 | 29.62 | 96.80 | 3.20 |
| kumar109.d | 6-5b | 1.50 | 30.6 | 309.2 | 90.65 | 4.84 | 1.57 | 57.20 | 22.08 | 8.13 | 0.70 | 1.34 | 0.00 | 0.00 | 0.50 | 7.48 | 62.11 | 30.42 | 96.77 | 3.23 |
| kumar110.d | 7a-2a | 1.40 | 41.9 | 255.9 | 102.96 | 3.87 | 1.20 | 26.74 | 15.53 | 8.83 | 0.69 | 14.17 | 0.00 | 0.00 | 0.34 | 5.94 | 68.80 | 25.26 | 58.50 | 41.50 |
| kumar111.d | 7a-2b | 1.40 | 41.3 | 259.9 | 103.08 | 3.89 | 1.37 | 27.81 | 14.89 | 8.42 | 0.74 | 14.69 | 0.00 | 0.00 | 0.36 | 6.21 | 69.60 | 24.19 | 58.68 | 41.32 |
| kumar112.d | 8-3a | 1.65 | 35.4 | 309.8 | 104.97 | 4.68 | 1.54 | 54.92 | 22.23 | 10.14 | 0.66 | 1.21 | 0.00 | 0.00 | 0.39 | 7.20 | 60.05 | 32.75 | 96.91 | 3.09 |
| kumar113.d | 8-3b | 1.60 | 32.9 | 349.5 | 109.90 | 4.56 | 1.44 | 56.89 | 21.57 | 9.68 | 0.64 | 1.21 | 0.00 | 0.00 | 0.38 | 6.93 | 61.65 | 31.43 | 97.20 | 2.80 |
| kumar114.d | 9-2a | 1.50 | 35.1 | 369.8 | 124.16 | 4.55 | 1.57 | 54.93 | 22.77 | 9.74 | 0.69 | 1.35 | 0.00 | 0.00 | 0.42 | 7.10 | 60.19 | 32.70 | 96.94 | 3.06 |
| kumar115.d | 9-2b | 1.44 | 31.9 | 436.7 | 133.42 | 4.47 | 1.57 | 54.19 | 24.03 | 9.30 | 0.68 | 1.33 | 0.00 | 0.00 | 0.43 | 7.01 | 59.35 | 33.63 | 96.82 | 3.18 |
| kumar116.d | 10-7a | 1.57 | 30.8 | 385.3 | 113.71 | 4.67 | 1.56 | 49.39 | 27.70 | 9.76 | 0.71 | 1.32 | 0.00 | 0.00 | 0.52 | 7.29 | 54.88 | 37.83 | 96.58 | 3.42 |
| kumar117.d | 10-7b | 1.33 | 29.4 | 394.9 | 111.25 | 4.50 | 1.40 | 46.17 | 30.19 | 10.91 | 0.64 | 1.29 | 0.00 | 0.00 | 0.47 | 6.83 | 51.70 | 41.47 | 96.76 | 3.24 |
| kumar118.d | 11-9a | 2.10 | 36.4 | 316.7 | 110.34 | 4.65 | 1.49 | 59.39 | 19.05 | 9.12 | 0.77 | 1.44 | 0.00 | 0.00 | 0.53 | 7.16 | 64.25 | 28.58 | 96.53 | 3.47 |
| kumar119.d | 11-9b | 1.99 | 36.9 | 293.3 | 103.60 | 4.68 | 1.53 | 60.35 | 18.66 | 8.72 | 0.77 | 1.41 | 0.00 | 0.00 | 0.52 | 7.24 | 65.16 | 27.60 | 96.78 | 3.22 |
| kumar120.d | 12-3a | 1.34 | 32.1 | 462.3 | 142.04 | 4.39 | 1.48 | 58.69 | 22.00 | 7.18 | 0.64 | 1.24 | 0.00 | 0.00 | 0.38 | 6.83 | 63.61 | 29.55 | 97.07 | 2.93 |
| kumar121.d | 12-3b | 1.39 | 32.6 | 476.7 | 148.52 | 4.37 | 1.46 | 59.31 | 22.17 | 7.04 | 0.68 | 1.28 | 0.00 | 0.00 | 0.39 | 6.83 | 63.56 | 29.61 | 96.95 | 3.05 |
| kumar122.d | 13a-2a | 1.41 | 19.5 | 408.5 | 76.32 | 6.18 | 3.17 | 59.65 | 16.92 | 3.54 | 1.51 | 1.69 | 1.68 | 1.00 | 0.00 | 12.75 | 64.35 | 22.54 | 92.19 | 7.81 |
| kumar123.d | blank2 | 2.76 | 0.0 | 8.3 | 0.00 | #DIV/0! | ##### | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! | ##### | #DIV/0! | ##### | #DIV/0! | ##### | ##### | ##### | ##### | #DIV/0! |
| kumar124.d | 13a-2b | 1.50 | 28.5 | 395.1 | 107.74 | 4.31 | 2.02 | 61.54 | 20.30 | 5.05 | 0.98 | 1.36 | 0.00 | 0.00 | 0.61 | 7.81 | 66.37 | 25.82 | 96.14 | 3.86 |
| kumar125.d | 15b-1a | 1.53 | 35.6 | 415.7 | 141.63 | 4.16 | 1.84 | 63.42 | 17.18 | 6.85 | 0.83 | 1.42 | 0.00 | 0.00 | 0.50 | 7.23 | 68.22 | 24.45 | 96.48 | 3.52 |
| kumar126.d | 15b-1b | 1.45 | 34.8 | 431.7 | 143.91 | 4.12 | 1.73 | 63.71 | 17.11 | 7.15 | 0.76 | 1.43 | 0.00 | 0.00 | 0.47 | 6.99 | 68.46 | 24.55 | 96.59 | 3.41 |
| kumar127.d | 16b-3a | 1.43 | 32.4 | 411.5 | 127.43 | 4.47 | 1.69 | 58.76 | 21.20 | 7.28 | 0.79 | 1.41 | 0.00 | 0.00 | 0.49 | 7.35 | 63.88 | 28.77 | 96.45 | 3.55 |
| kumar128.d | 16b-3b | 1.35 | 32.9 | 412.3 | 129.80 | 4.51 | 1.67 | 58.52 | 21.06 | 7.68 | 0.75 | 1.38 | 0.00 | 0.00 | 0.49 | 7.33 | 63.63 | 29.04 | 96.53 | 3.47 |
| kumar129.d | 17a-3a | 1.47 | 33.6 | 493.9 | 158.68 | 4.49 | 1.70 | 58.72 | 21.06 | 7.45 | 0.78 | 1.25 | 0.00 | 0.00 | 0.42 | 7.32 | 63.63 | 28.96 | 96.83 | 3.17 |
| kumar130.d | 17a-3b | 1.35 | 34.4 | 450.3 | 148.28 | 4.42 | 1.64 | 57.50 | 21.96 | 8.27 | 0.69 | 1.25 | 0.00 | 0.00 | 0.43 | 7.07 | 62.40 | 30.54 | 96.95 | 3.05 |

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| kumar131.d | 18a-4a | 1.59 | 33.2 | 428.1 | 135.99 | 4.72 | 1.60 | 52.51 | 25.17 | 9.39 | 0.67 | 1.28 | 0.00 | 0.00 | 0.42 | 7.31 | 57.74 | 34.95 | 96.92 | 3.08 |
| kumar132.d | 18a-4b | 1.42 | 32.4 | 444.0 | 137.55 | 4.75 | 1.68 | 51.78 | 25.46 | 9.40 | 0.71 | 1.32 | 0.00 | 0.00 | 0.41 | 7.46 | 57.10 | 35.30 | 96.78 | 3.22 |
| kumar133.d | 21-2a | 1.34 | 35.3 | 359.7 | 121.40 | 4.33 | 1.43 | 57.81 | 22.39 | 8.49 | 0.63 | 1.27 | 0.00 | 0.00 | 0.43 | 6.69 | 62.27 | 31.04 | 97.11 | 2.89 |
| kumar134.d | 21-2b | 1.45 | 35.7 | 362.8 | 123.98 | 4.36 | 1.45 | 56.57 | 23.50 | 8.38 | 0.69 | 1.26 | 0.00 | 0.00 | 0.41 | 6.80 | 60.95 | 32.25 | 96.98 | 3.02 |
| kumar135.d | 22-6a | 1.54 | 35.7 | 252.0 | 86.12 | 4.38 | 1.51 | 53.25 | 24.36 | 10.97 | 0.61 | 1.13 | 0.00 | 0.00 | 0.35 | 6.72 | 57.96 | 35.33 | 97.44 | 2.56 |
| kumar136.d | 22-6b | 1.48 | 34.2 | 246.0 | 80.43 | 4.68 | 1.61 | 51.72 | 25.54 | 10.93 | 0.60 | 1.13 | 0.00 | 0.00 | 0.35 | 7.10 | 56.42 | 36.47 | 97.45 | 2.55 |
| kumar137.d | 23a-1a | 1.72 | 32.0 | 462.8 | 141.56 | 4.63 | 1.98 | 65.23 | 16.39 | 5.06 | 0.89 | 1.42 | 0.00 | 0.00 | 0.55 | 7.95 | 70.08 | 21.97 | 96.08 | 3.92 |
| kumar138.d | 23a-1b | 1.32 | 31.1 | 481.7 | 143.47 | 4.59 | 1.96 | 65.42 | 16.69 | 5.35 | 0.92 | 1.48 | 0.00 | 0.00 | 0.56 | 7.94 | 69.49 | 22.48 | 96.15 | 3.85 |
| kumar139.d | 43 DGAT Quantum t1-1a | 1.79 | 42.7 | 317.8 | 129.78 | 4.41 | 1.91 | 66.08 | 15.85 | 5.26 | 0.87 | 1.23 | 0.00 | 0.00 | 0.52 | 7.61 | 70.91 | 21.48 | 96.59 | 3.41 |
| kumar140.d | 43 DGAT Quantum T1 b | 1.55 | 38.1 | 319.8 | 116.61 | 4.59 | 1.84 | 65.58 | 15.91 | 5.33 | 0.85 | 1.26 | 0.00 | 0.00 | 0.53 | 7.73 | 70.57 | 21.69 | 96.41 | 3.59 |
| kumar141.d | 48 DGAT Quantum T1 a | 2.47 | 32.0 | 311.4 | 95.44 | 4.93 | 1.82 | 61.22 | 18.73 | 6.32 | 0.88 | 1.25 | 0.00 | 0.00 | 0.50 | 8.13 | 66.83 | 25.05 | 96.61 | 3.39 |
| kumar142.d | 48 DGAT Quantum T1 b | 1.76 | 31.5 | 333.3 | 100.34 | 5.08 | 1.80 | 60.46 | 18.94 | 6.24 | 0.85 | 1.23 | 0.00 | 0.00 | 0.51 | 8.22 | 66.37 | 25.41 | 96.65 | 3.35 |
| kumar143.d | 49 DGAT Quantum T1 a | 2.63 | 41.4 | 266.8 | 105.70 | 4.81 | 1.75 | 63.17 | 17.12 | 6.99 | 0.86 | 1.10 | 0.00 | 0.00 | 0.46 | 7.76 | 67.66 | 24.58 | 96.77 | 3.23 |
| kumar144.d | 49DGAT Quantum T1 b | 1.71 | 38.3 | 289.4 | 106.10 | 4.97 | 1.61 | 60.73 | 18.24 | 7.78 | 0.73 | 1.05 | 0.00 | 0.00 | 0.40 | 7.64 | 65.67 | 26.70 | 96.82 | 3.18 |
| kumar145.d | 5a Batch 2 DGAT a | 2.26 | 38.3 | 402.8 | 147.74 | 4.47 | 1.89 | 61.99 | 19.46 | 5.99 | 0.90 | 1.34 | 0.00 | 0.00 | 0.59 | 7.63 | 66.34 | 26.02 | 96.11 | 3.89 |
| kumar146.d | 5a Batch 2 DGAT b | 1.78 | 38.7 | 376.3 | 139.19 | 4.70 | 2.05 | 61.21 | 19.30 | 6.28 | 0.98 | 1.37 | 0.00 | 0.00 | 0.58 | 8.10 | 65.71 | 26.19 | 95.99 | 4.01 |

| GC 6890 front injector Nov. | | Batch Nov.27 and 28 | | | average of 2 |
|---|---|---|---|---|---|
| Vial # | Sample | Wt. 12 seeds mg | GC Chart # | %oil | |
| 1 | Blank | | 99 | | |
| 2 | Quantum Control a | 38.58 | 100 | 37.21 | 37.34 |
| 3 | Quantum Control b | 34.93 | 101 | 37.46 | |
| 4 | 3-2 a | 41.13 | 102 | 26.78 | 26.52 |
| 5 | 3-2 b | 36.7 | 103 | 26.25 | |
| 6 | 4-2 a | 44.4 | 104 | 28.91 | 29.64 |
| 7 | 4-2 b | 45.84 | 105 | 30.37 | |
| 8 | 5-2 a | 33.1 | 106 | 29.71 | 30.20 |
| 9 | 5-2 b | 31.48 | 107 | 30.68 | |
| 10 | 6-5 a | 35.53 | 108 | 29.03 | 29.13 |
| 11 | 6-5 b | 37.1 | 109 | 29.23 | |
| 12 | 7a-2 a | 30.71 | 110 | 40.14 | 39.99 |
| 13 | 7a-2 b | 31.19 | 111 | 39.83 | |
| 14 | 8-3 a | 37.18 | 112 | 34.01 | 32.71 |
| 15 | 8-3 b | 41.94 | 113 | 31.4 | |
| 16 | 9-2 a | 44.38 | 114 | 33.6 | 32.11 |
| 17 | 9-2 b | 52.4 | 115 | 30.62 | |
| 18 | 10-7 a | 46.24 | 116 | 29.69 | 29.10 |
| 19 | 10-7 b | 47.39 | 117 | 28.51 | |
| 20 | 11-9 a | 38 | 118 | 34.79 | 35.01 |
| 21 | 11-9 b | 35.1 | 119 | 35.22 | |
| 22 | 12-3 a | 55.47 | 120 | 30.67 | 30.89 |
| 23 | 12-3 b | 57.2 | 121 | 31.11 | |
| 24 | 13a-2 a | 49.02 | 122 | 31.3 | 30.99 |
| 25 | control blank -1 | | 123 | | |
| 26 | 13a-2 b | 47.41 | 124 | 27.98 | |
| 27 | 15b-1 a | 49.88 | 125 | 33.96 | 33.53 |
| 28 | 15b-1 b | 51.8 | 126 | 33.1 | |
| 29 | 16b-3 a | 49.38 | 127 | 30.91 | 31.15 |
| 30 | 16b-3 b | 49.47 | 128 | 31.39 | |
| 31 | 17a-3 a | 59.27 | 129 | 30.91 | 31.92 |
| 32 | 17a-3 b | 54.04 | 130 | 32.93 | |
| 33 | 18a-4 a | 51.37 | 131 | 31.84 | 31.57 |
| 34 | 18a-4 b | 53.28 | 132 | 31.3 | |
| 35 | 21-2 a | 43.16 | 133 | 33.72 | 33.92 |
| 36 | 21-2 b | 43.54 | 134 | 34.12 | |
| 37 | 22-6 a | 30.24 | 135 | 34.34 | 33.76 |
| 38 | 22-6b | 29.52 | 136 | 33.18 | |
| 39 | 23a-1 a | 55.53 | 137 | 30.38 | 30.08 |
| 40 | 23a-1 b | 57.8 | 138 | 29.78 | |
| 41 | 43 DGAT Quantum T1 a | 38.14 | 139 | 40.43 | 38.36 |
| 42 | 43 DGAT Quantum T1 b | 38.37 | 140 | 36.28 | |
| 43 | 48 DGAT Quantum T1 a | 37.37 | 141 | 30.49 | 30.20 |
| 44 | 48 DGAT Quantum T1 b | 40 | 142 | 29.91 | |
| 45 | 49 DGAT Quantum T1 a | 32.01 | 143 | 39.34 | 38.02 |
| 46 | 49 DGAT Quantum T1 b | 34.73 | 144 | 36.69 | |
| 47 | 5a Batch 2 DGAT a | 48.33 | 145 | 36.52 | 36.75 |
| 48 | 5a Batch 2 DGAT b | 45.15 | 146 | 36.98 | |

| Run # | Row #, Seed | 17:0 | % Oil | mg oil /100 seeds | mg FA's /100 seeds | % 14:0 | % 16:0 | % 16:1c9 | % 16:2 | % 16:3 | % 18:0 | % 18:1c9 | % 18:1c11 | % 18:2 | % 18:3 | % 20:0 | % 20:1c11 | % 20:1c13 | % 20:2 | % 22:0 | % 22:1c13 | % 22:1c15 | % 24:0 | % 24:1c15 | Total | % Sats | % Mono | % Poly | Wt % LCFA | Wt % VLCFA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ah340 d | Con | 1.59 | 31.7 | 439.5 | 130.42 | 0.00 | 4.50 | 0.19 | 0.07 | 0.22 | 1.37 | 57.49 | 2.45 | 21.66 | 8.61 | 0.58 | 1.39 | 0.00 | 0.15 | 0.42 | 0.04 | 0.00 | 0.00 | 0.04 | 99.38 | 7.21 | 61.84 | 30.95 | 99.76 | 3.24 |
| ah341 d | 3'-1 | 1.34 | 28.0 | 465.2 | 132.77 | 0.00 | 4.72 | 0.23 | 0.07 | 0.24 | 2.00 | 51.39 | 3.02 | 25.66 | 8.80 | 0.78 | 1.24 | 0.00 | 0.16 | 0.84 | 0.04 | 0.03 | 0.00 | 0.03 | 0.06 | 99.34 | 7.85 | 56.00 | 36.15 | 99.87 | 3.13 |
| ah342 d | 4'-1 | 1.38 | 27.9 | 521.6 | 139.22 | 0.00 | 4.89 | 0.22 | 0.07 | 0.19 | 1.58 | 51.90 | 2.48 | 26.83 | 8.73 | 0.63 | 1.12 | 0.00 | 0.16 | 0.44 | 0.03 | 0.00 | 0.03 | 0.06 | 99.31 | 8.06 | 53.19 | 28.19 | 99.87 | 3.43 |
| ah343 d | 5'-1 | 1.36 | 27.0 | 439.8 | 113.56 | 0.00 | 4.82 | 0.23 | 0.12 | 0.21 | 1.98 | 59.16 | 2.25 | 20.77 | 6.93 | 0.61 | 1.20 | 0.00 | 0.13 | 0.55 | 0.03 | 0.00 | 0.00 | 0.04 | 98.31 | 8.06 | 63.19 | 28.19 | 96.87 | 3.43 |
| ah344 d | 6'-2 | 1.22 | 28.9 | 440.4 | 121.82 | 0.00 | 4.91 | 0.22 | 0.09 | 0.24 | 1.87 | 58.80 | 2.43 | 20.67 | 7.75 | 0.66 | 1.27 | 0.00 | 0.15 | 0.43 | 0.03 | 0.03 | 0.00 | 0.04 | 99.29 | 7.95 | 63.12 | 28.94 | 96.98 | 3.32 |
| ah345 d | 7a-1 | 2.24 | 34.7 | 411.8 | 136.69 | 0.00 | 4.64 | 0.20 | 0.10 | 0.20 | 1.63 | 43.80 | 2.02 | 20.07 | 9.17 | 0.50 | 11.06 | 0.29 | 0.38 | 0.19 | 4.84 | 0.05 | 0.00 | 0.07 | 99.30 | 7.07 | 62.88 | 30.05 | 81.82 | 18.19 |
| ah346 d | 8'-1 | 1.18 | 31.9 | 452.7 | 144.74 | 0.00 | 4.38 | 0.21 | 0.09 | 0.18 | 1.54 | 60.48 | 2.20 | 22.33 | 5.79 | 0.56 | 1.07 | 0.00 | 0.15 | 0.32 | 0.03 | 0.00 | 0.04 | 0.04 | 99.41 | 7.13 | 64.23 | 28.62 | 97.20 | 2.80 |
| ah347 d | 9'-1 | 1.19 | 32.6 | 515.6 | 160.47 | 0.00 | 4.58 | 0.19 | 0.09 | 0.21 | 1.62 | 57.62 | 2.32 | 22.63 | 7.48 | 0.64 | 1.15 | 0.00 | 0.14 | 0.39 | 0.03 | 0.02 | 0.02 | 0.04 | 99.37 | 7.57 | 61.63 | 30.81 | 96.83 | 3.07 |
| ah348 d | Con2 | 1.22 | 34.7 | 430.5 | 143.09 | 0.00 | 4.47 | 0.18 | 0.09 | 0.22 | 1.38 | 55.84 | 2.48 | 22.61 | 9.47 | 0.56 | 1.30 | 0.00 | 0.15 | 0.39 | 0.03 | 0.03 | 0.03 | 0.04 | 99.38 | 7.13 | 59.94 | 32.80 | 96.83 | 3.17 |
| ah349 d | 10'-6 | 1.57 | 33.2 | 497.8 | 158.30 | 0.00 | 4.61 | 0.19 | 0.07 | 0.24 | 1.61 | 52.19 | 2.60 | 25.31 | 8.64 | 0.67 | 1.28 | 0.00 | 0.17 | 0.47 | 0.04 | 0.00 | 0.03 | 0.05 | 99.53 | 7.98 | 58.61 | 35.71 | 96.66 | 3.34 |
| ah350 d | 11'-1 | 1.24 | 33.4 | 394.7 | 126.12 | 0.00 | 5.07 | 0.22 | 0.11 | 0.26 | 1.50 | 57.40 | 2.28 | 21.48 | 8.84 | 0.65 | 1.15 | 0.00 | 0.09 | 0.46 | 0.00 | 0.00 | 0.00 | 0.00 | 99.53 | 7.91 | 61.32 | 30.78 | 97.17 | 2.83 |
| ah351 d | 12'-2 | 1.48 | 28.8 | 575.2 | 186.14 | 0.00 | 4.82 | 0.21 | 0.08 | 0.21 | 1.60 | 54.17 | 2.60 | 26.14 | 7.60 | 0.59 | 1.09 | 0.00 | 0.15 | 0.35 | 0.03 | 0.00 | 0.02 | 0.04 | 99.39 | 7.40 | 58.37 | 34.23 | 97.13 | 2.87 |
| ah352 d | 17a-1 | 1.44 | 30.2 | 631.9 | 182.85 | 0.00 | 4.81 | 0.23 | 0.10 | 0.23 | 1.72 | 54.27 | 2.70 | 24.50 | 8.44 | 0.65 | 1.14 | 0.02 | 0.15 | 0.41 | 0.03 | 0.03 | 0.03 | 0.04 | 99.31 | 7.76 | 58.71 | 33.49 | 96.79 | 3.21 |
| ah353 d | 18a-1 | 1.29 | 31.8 | 488.9 | 141.93 | 0.00 | 5.00 | 0.24 | 0.09 | 0.19 | 1.68 | 51.73 | 3.05 | 25.64 | 8.38 | 0.58 | 1.07 | 0.00 | 0.16 | 0.32 | 0.00 | 0.00 | 0.03 | 0.04 | 99.46 | 7.84 | 56.36 | 35.79 | 97.27 | 2.73 |
| ah354 d | 21'-1 | 1.55 | 32.9 | 497.8 | 156.75 | 0.00 | 4.56 | 0.19 | 0.10 | 0.19 | 1.78 | 55.71 | 2.58 | 24.48 | 7.41 | 0.65 | 1.17 | 0.00 | 0.15 | 0.38 | 0.03 | 0.03 | 0.03 | 0.04 | 99.43 | 7.67 | 59.94 | 32.39 | 96.95 | 3.05 |
| ah355 d | 22'-1 | 1.40 | 34.1 | 271.0 | 88.51 | 0.00 | 4.65 | 0.29 | 0.08 | 0.21 | 1.50 | 46.85 | 3.26 | 28.30 | 11.31 | 0.50 | 1.03 | 0.00 | 0.16 | 0.33 | 0.00 | 0.00 | 0.00 | 0.06 | 99.41 | 7.32 | 51.56 | 41.12 | 97.27 | 2.73 |